ates the adhesion of *S. typhimurium* to murine Peyer's patches [21]. LPF was first described in *S. typhimurium*, and is found in numerous pathogenic *E. coli* strains [22]. Although its specific receptor remains unclear, extracellular matrix proteins (ECMs), which comprise an interlocking mesh of fibrous proteins and glycosaminoglycans, may act as a receptor for LPF of enterohemorrhagic *E. coli* O157:H7 (Fig. 3). ECMs are modified by various carbohydrate moieties, and the addition of mannose inhibits LPF-ECM interaction. Then mannose-containing carbohydrates may participate in bacterial adhesion by LPF [23].

In some cases, type 4 fimbriae are encoded on plasmids. Such plasmids frequently encode virulence factors for host bacteria, and are therefore called "virulence plasmids" [24]. PEF is required for bacterial attachment to intestinal epithelial cells. It specifically binds to trisaccharide Galβ1-4(Fucα1-3)GlcNAc, also known as the Lewis X (Le^X) blood group antigen (Fig. 3) [13]. The Le^X antigen is defined by the presence of a terminal Galβ1-4(Fucα-3)GlcNAc moiety on saccharide chains of glycoproteins or glycosphingolipids; in the human intestine, it is expressed mainly in crypt epithelial cells [25]. *S. typhimurium* possesses PEF as an adhesin that binds to a crypt-specific histo-blood group antigen that may be relevant to the pathogenesis of human infections. Abundant crypt abscesses are commonly found in *S. typhimurium* patients, raising the possibility that the pathogen may bind to human crypt epithelium at a later stage of infection. In a situation where Peyer's patches are unavailable because of an inflammatory reaction, *Salmonella* can colonize at the crypt epithelium remaining intact and persist on the surface of the host intestinal tract [13, 25].

On the other hand, some type 4 fimbriae participate in "fimbria-mediated (pilus-mediated) conjugal transfer" of so-called "conjugative plasmids". Conjugative plasmids can also be virulence plasmids if they encode not only the structural genes of the fimbriae but also other virulence factors, such as a drug resistance gene. These conjugative plasmids spread to other bacteria by horizontal transfer, and type 4 fimbriae encoded on the plasmids play an important role in this event. For example, the R64 plasmid, which encodes the *pilV* gene and engages in the adhesion of type 4 fimbriae, recognizes the di-saccharide moiety of bacterial surface polysaccharides (the core oligosaccharide or O-antigen unit of lipopolysaccharides, a unique structure of the bacterial cell surface) and determines the recipient bacteria of the conjugal transfer [26, 27].

Categorized as π-fimbriae, the std fimbriae are well conserved among *S. enterica* serotypes but absent from related bacterial species (Fig. 2). Std fimbriae recognize and bind the H type 2 histo-blood group oligosaccharide, the terminal Fucα1-2Galβ1-4GlcNAc moiety. This structure represents the H type 2 oligosaccharide of the O blood group antigen [14]. The H type 2 oligosaccharide of the O blood group antigen moiety is expressed as part of the mucin-type sugar chains of glycoproteins in the host cell. The terminal Fucα1-2 moiety of H type 2 oligosaccharide of the O blood group antigen is essential for the recognition of Std fimbriae (Fig. 3).

Carbohydrate molecules act not only as "anchors" for pathogens but also as the determinants of host and tissue specificity. The variety of adhesion factors carried by a bacterium reflects its pathogenic profile, magnitude of virulence, host specificity, and tissue specificity. In the case of *Salmonella* and assortative bacteria, the FimH adhesins show amino acid sequence diversity. This diversity in FimH structure results in the variation in affinity profiles. *E. coli* FimH shows a high affinity for aromatic α-mannosides as well as Manα1-3 structures. On the other hand, the FimH of *Salmonella* species shows a high affinity for α-mannosides and a low affinity for aromatic α-mannosides [19]. In the case of *Salmonella*, allelic variation of FimH adhesion directs not only host cell-specific recognition but also distinctive binding to mammalian and avian receptors. This allele-specific binding profile parallels the host specificity of the respective FimH-expressing pathogen [28]. Similarly, the Lewis b (Le^b) blood group phenotype in combination with secretor status may hinder colonization of *Helicobacter pylori* in certain populations [29]. *H. pylori* express blood group antigen b-binding adhesion (BabA), and BabA binds to Le^b antigens. *Salmonella* and assortative bacteria contain various adhesion factors, including several kinds of fimbriae, which contribute to bacterial virulence; however, analyses of their specific receptor moieties and functions are not yet complete [13, 15].

Carbohydrate moieties on the surface of pathogens are also recognized by hosts and trigger host defense mechanisms. The bacterial surface is covered with various kinds of carbohydrates. For gram-negative bacteria, including *Salmonella*, the major carbohydrate component of the bacterial surface is lipopolysaccharide (LPS). LPS is categorized as a glycolipid, and is a major component of the bacterial outer membrane. Because the saccharide moieties of LPS differ structurally from mammalian carbohydrates, they function as targets of the host immune response. To avoid this host immune response, the LPS of some bacteria, for example *C. jejuni*, is structurally similar to the glycosphingolipids of gangliosides [30, 31]. Similarly, the LPS of most *H. pylori* strains expresses the Le^a and Le^b antigens [29].

Interestingly, the carbohydrate on the surface of the

host cell itself can be involved in the host defense mechanism. The *Salmonella* flagella component FliC contributes to bacterial attachment to the host cell by interacting with ganglioside molecules on the surface of the host cell, but gangliosides also act as co-receptors for *Salmonella* enterica FliC and promote FliC induction of the human innate immune response [32]. Gangliosides, i.e. sialic acid-containing glycosphingolipids, are ubiquitous components of eukaryotic cell membranes that have been identified as receptors for bacterial toxins and viruses. An *in vitro* assay showed that a nonflagellated mutant of *S. enteritidis*, constructed by disrupting the *fliC* gene, was about 50-fold less invasive than the wild-type strain, but bacterial adherence was unaffected [33]. At the attachment of *Salmonella enteritidis* FliC to the host cell surface, gangliosides thus function as receptors.

On the other hand, the flagella component protein FliC induces the host innate immune response by binding to Toll-like receptor 5 of the host cell, and gangliosides react as co-receptors with TLR5 on the FliC-induced response. An *in vitro* assay showed that the incorporation of exogenous ganglioside GD1a into the Caco-2 cell membrane increased the effect of FliC. Incubation of Caco-2 cells with a glucosylceramide synthase inhibitor reduced the innate immune response stimulated by FliC [32].

Interaction between Enteric Protozoa and Carbohydrates

Human enteropathogenic protozoas include the apicomplexans *Toxoplasma gondii* and *Cryptosporidium* as well as *Giardia* and *Entamoeba histolytica*. They are all zoonotic pathogens that invade and colonize their target tissues in the alimentary tract of the human host. They form hard cysts that resist degradation in the stomach. Host-derived proteases and low pH trigger their excystation [34].

In this section, we describe the role of carbohydrates in *Toxoplasma gondii* invasion of intestinal epithelial cells. The ability of *T. gondii* to infect Chinese hamster ovary (CHO) cells deficient in sialic acids was reduced by 26.9% compared to wild-type cells, indicating that sialic acid is critical for attachment and invasion of *T. gondii* (Fig. 4) [35]. *T. gondii* microneme protein 1 (TgMIC1) forms a macromolecular complex with TgMIC4 and TgMIC6. Single deletion of the *TgMIC1* gene significantly decreases the invasion of host cells, suggesting an essential role for TgMIC1 in host cell attachment and invasion of *T. gondii* [36]. Structural analysis of TgMIC1 revealed a novel cell-binding motif called microneme adhesive repeat region (MARR), which provides a specialized structure for glycan

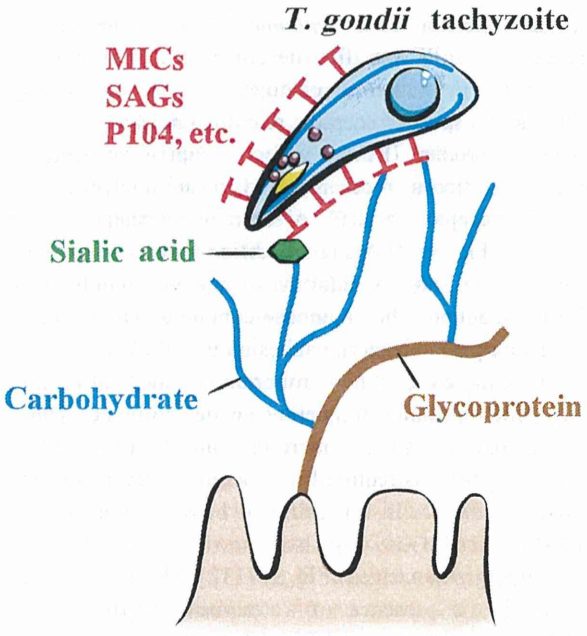

Fig. 4. Schematic image of enteric pathogen (in this case *T. gondii*) invasion showing secreted proteins attached to carbohydrates on host cells.
Some of the proteins from *T. gondii* tachyzoite (e.g., MICs, SAGs, P104) can be secreted or membrane-bound and attached to sialic acids, carbohydrates and glycoproteins during invasion.

discrimination [37]. Carbohydrate microarray analyses showed that TgMIC13, TgMIC1 and its homologue *Neospora caninum* MIC1 share a preference for α2-3- over α2-6-linked sialyl-N-acetyllactosamine sequences [38]. P104, a PAN/apple domain-containing protein expressed at the apical end of the extracellular parasite, functions as a ligand in the attachment of *T. gondii* to chondroitin sulfate and other receptors on the host cell, facilitating invasion by the parasite (Fig. 4) [39].

T. gondii display GPI-anchored surface proteins identified as surface antigen glycoprotein (SAG) 1 related sequences (SRS) [40]. SAG1, SAG2A and SAG3 have some capacity for host cell attachment through glycan recognition (Fig. 4) [41, 42]. SAG3 binds to sulfated proteoglycans such as heparin, fucoidan, and dextran sulfate with high affinity [43]. Targeted disruption of SAG3 significantly reduces host cell binding of *T. gondii* [41].

E. histolytica fibronectin receptor (EhFNR) shows 99% homology to the intermediate subunit-2 of the Gal/GalNAc-specific lectin [44]. Electron microscopy revealed the close association of a purified EhFNR complex to adhesion plates and phagocytic invaginations. Lipid rafts participate in interactions between *E. histolytica* and the host

extracellular matrix, and it appears that raft-associated Gal/GalNAc lectin serves as a collagen receptor [45].

Cryptosporidium parvum surface receptors, GP900 and proteolytic fragments of the 60-kDa precursor protein, GP40 and GP15, are characterized as mucin-like and heavily O-glycosylated proteins [46–48]. The GP900 and GP40 of sporozoites and merozoites have carbohydrate residues that are bound by αGalNAc-specific lectins, suggesting that αGalNAc residues are involved in the attachment of parasites to host cells via adherence to internal mucus.

Apicomplexan protozoan parasites also induce host innate immune responses via the carbohydrate molecules present on their cell surface [49]. Glycosylphosphatidylinositol (GPI) protein anchors are abundant in the membranes of tachyzoites and other apicomplexan protozoan parasites including *Trypanosoma*, *Leishmania* and *Plasmodium* spp., where they can serve as ligands for innate recognition [50]. The GPI moieties of *T. cruzi* and *P. falciparum* were found to be TLR2 ligands [51, 52], and *T. gondii* both stimulate cytokine production in macrophages and serve as TLR2 as well as TLR4 agonists. In the case of *T. gondii*, GPI induces TNF-α production in macrophages through the activation of the transcription factor NF-κB [53].

Comparison of the Interactions between Enteric Microbes and Carbohydrates

Carbohydrates serve as receptors for infections by viruses, bacteria and protozoa, but the usage of carbohydrates by these microbes varies depending on the microbe. At the initial infection step, these organisms do not simply utilize the electrical forces created by the positive and negative charges of the carbohydrates; rather, they make use of other systems in certain instances. One similarity shared by all three microbes regarding their interactions with carbohydrates, however, is that heparan sulfate plays an important role at entry or invasion of the host cell.

Blood group antigen oligosaccharides are highly expressed in the gastrointestinal epithelium [54]. However, there are individual differences in terms of the presence of these antigens. In addition, there are individual differences in sensitivity to pathogens that recognize and bind to blood group antigens, such as norovirus and *H. pylori*. These individual differences in antigen expression profiles benefit the survival of the host species because the risk of an attack by a fatal virulent pathogen may be decreased to avoid extinction.

The Structure of Carbohydrates on the Surface of the Gastric and Intestinal Epithelium

A large array of glycoproteins, glycolipids and proteoglycans decorate the surface of animal cells. These glycoconjugates mediate many fundamental cellular processes, including cell-cell and cell-matrix adhesion, motility, growth and signaling [55–57]. Mucosal tissues represent the site of infection or route of access for most parasites, including viruses, bacteria and protozoa [58]. On the surface of the mucosal tissues of the gastrointestinal tracts, various carbohydrate moieties are present and play a crucial role in infection.

Mucosal surfaces are coated with a layer of viscous mucus that ranges in thickness from 300 μm in the stomach to 700 μm in the intestine [59–61]. Mucin glycoproteins from mucus-producing cells in the epithelium or submucosal glands are the major macromolecular constituent of mucus and are responsible for the viscous properties of the mucus gel. In addition to forming a relatively impervious gel, which acts as a lubricant, a physical barrier and a trap for microbes, mucus provides a matrix for a rich array of antimicrobial molecules. Underneath the mucus layer, the cells present a dense forest of highly diverse glycoproteins and glycolipids, which form the glycocalyx. Membrane-anchored cell-surface mucin glycoproteins are a major constituent of the glycocalyx in all mucosal tissues. The oligosaccharide moieties of the molecules that form the glycocalyx and the mucus layer are highly diverse, and the average turnover time of the human jejunal glycocalyx is 6–12 h [62]. Consequently, both the secreted and adherent mucosal barriers are constantly renewed and can rapidly adjust to changes in the environment, for example, in response to microbial infection.

Epithelial mucins are a heterogenous family of large complex glycoproteins containing a dense array of O-linked carbohydrates typically comprising over 70% of their mass. The carbohydrate structures present on mucosal surfaces vary according to cell lineage, tissue location and developmental stage [58]. Mucin glycosylation can alter in response to mucosal infection and inflammation, and this may be an important mechanism for unfavorable changes in the niche occupied by mucosal pathogens. The O-linked glycans of muchin proteins contain 1–20 residues, which occur both as linear and branched structures [58].

In addition to the O-linked glycans, mucins contain a smaller number of N-linked oligosaccharides, which have been implicated in folding, oligomerization (MUC2) and surface localization (MUC17) [63–65]. The terminal structures of mucin oligosaccharides are highly heterogeneous

and vary between and within species as well as between and even within tissues. The array of oligosaccharide structures on individual mucin molecules is also somewhat determined by stochastic events as the mucin protein moves through the Golgi apparatus [66]. The secreted mucins themselves likely function as decoys for adhesins that have been evolved by pathogens to engage the cell surface, as the mucins express many of the oligosaccharide structures found on the cell surface and are constitutively produced in large amounts, constantly washing the mucosal surfaces [58].

Proteoglycans are present on the cell surface [67] and are also components of glycocalyx. Glycosaminoglycan chains are composed of highly sulfated saccharides that give the cell surface a potent negative charge. One of the prototypical membrane proteoglycans is syndecan-1, which carries conserved attachment sites for glycosaminoglycan chains [67]. The syndecans exemplify hybrid proteoglycans because they contain mixtures of the two major types of glycosaminoglycan chains, heparan sulfate and chondroitin sulfate. The other major family of membrane proteoglycans is the glypicans, which contain GPI anchors in a tissue-specific and temporally regulated manner. Their presence in the basolateral membranes of polarized cells varies [68].

Glycolipids are also a component of the cell membrane. A large variety of glycolipids is present on the surface of animal cells. The carbohydrate moieties vary, and each glycolipid may exhibit a special function, as an annular lipid, surface receptor marker or matrix lipid. For brain and neuronal cells, gangliosides (sialic acid-bearing glycolipids) are the major cell surface determinants [69]. Glycolipids function as the receptor for various biologic factors and also as the receptor for various pathogens. They are present at the undermost part of the glycocalyx. Pathogens can recognize the glycolipids, directly bind to the cell membrane, and invade the host cell. Glycolipids also function as receptors for certain effector molecules, such as bacterial toxins, produced by pathogens and directly react with the host cell. For example, cholera toxin binds to ganglioside GM1 [70].

Thus, for pathogens living in the outer mucus layer, it is difficult to make contact with the surface of normal epithelial cells because of the huge amount of mucin that functions as a "decoy" or "physical barrier". Mucosal pathogens have, therefore, developed mechanisms to subvert these defense mechanisms of the mucosal layer. On the other hand, intestinal M cells, specifically designed to capture and present microbes to the underlying lymphoid tissue, can be regarded as a "hole" in the mucin barrier. The dome epithelium lacks goblet cells and therefore does not produce gel-forming mucins. Their apical cell surface has only sparse microvilli and an apparently thin glycocalyx [71, 72].

M cells are specialized epithelial antigen-transporting cells that constitute a minor proportion (5%~10% in humans and mice) of the follicle-associated epithelium that covers the lymphoid follicles of organized gut-associated lymphoid tissue such as Peyer's patches [73–76]. Glycoprotein 2 (GP2) was identified as an M cell-specific molecule [77]. The GP2 expressed on M cells functions as a bacterial uptake receptor [77]. GP2 recognizes FimH, a major component of the type 1 fimbriae, which binds to certain glycoproteins on mammalian cells in a mannose-dependent manner [78].

Consequently, even though M cells constitute only a very small percentage of mucosal epithelial cells, they are the major point of attachment and/or entry used by numerous mucosal pathogens including bacteria (e.g., *S. typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica* and *Vibrio cholerae*), viruses (e.g., reovirus, HIV-1 and polio virus) and parasites (e.g., *Cryptosporidia*) [72, 79, 80].

The Association between Carbohydrates and Microbial Infection

During cell–pathogen interactions (i.e., infection and/or invasion), carbohydrates function as receptors for various pathogens. On the other hand, carbohydrates (glycoconjugates) can also function as a barrier to infection. On the surface of mucosal tissue, the glycocalyx physically prevents microbes from accessing the cell membrane. Some glycoconjugates, a component of the glycocalyx, contain carbohydrate structures that are recognized by pathogens. Mucins often contain oligosaccharide moieties that correspond to the receptor for various pathogens. On the surface of the mucosal layer, microbes binds to these receptor moieties and are captured at the mucus layer, which consequently blocks the infection. Moreover, when secretory mucins containing receptor carbohydrate structures "trap" pathogens, the pathogens are also carried away. M cells are specialized epithelial antigen-transporting cells scattered in the follicle-associated epithelium that covers the gut lymphoid follicles such as Peyer's patches. M cells can efficiently engulf particles as large as bacteria; however, the mucus layer of M cells and the surrounding area is relatively thin. Glycoconjugates such as GP2 are expressed on the surface of M cells and function as receptors for bacterial attachment [74]. In the case of the host-parasite interaction, the various kinds of glycoconjugates sometimes function as receptors for the invading pathogens, but they can also function as barriers

and traps for the host defense system.

Future Perspectives

In recent years, the damage caused by enteric pathogens, especially norovirus and *Salmonella*, has expanded through the food chain [4, 5, 81]. These pathogens cause food poisoning in humans and gastrointestinal diseases in animals all over the world. Even today, they are often responsible for large-scale outbreaks of food poisoning. Therefore, the prevention and treatment of infections caused by these pathogens is essential.

In this review, we discussed the interaction between host cells and microbes such as viruses, bacteria and protozoa that involve carbohydrates such as sialic acids, heparan sulfate, and the carbohydrate moieties of ABH and Lewis antigens, mannose components, ECMs and Le^X. The development and use of drugs that target these carbohydrates is anticipated, even though the microbes vary widely and have different modes of infection. Accordingly, when an anti-microbial drug is developed on the basis of the interaction between a microbe and a carbohydrate, host cell modification of the drug's structure and/or inhibition of the mode of infection will need to be individualized while still taking advantage of the similarities between interactions.

Moreover, the host gastrointestinal tract cell surface, which is the object of microbial infection, is composed of glycoproteins, glycolipids, and proteoglycans. These molecules are potential targets for carbohydrate drugs used in the treatment of infectious diseases.

Oseltamivir and zanamivir are neuraminidase inhibitors that competitively inhibit the activity of the viral neuraminidase on the sialic acid that is found on glycoproteins on the surface of host cells [82]. By blocking the activity of this enzyme, they prevent new viral particles being released from infected cells.

There are various kinds of polysaccharides on the surface of bacteria. Lipoteichoic acid (LTA), a type of glycolipid, is a component of the bacterial cell wall of gram-positive bacteria. Studies have shown that LTA stimulates the immune system [83, 84]. Recently, LTA has been studied for use as a novel kind of biologically active substance.

Recently, sulfated polysaccharides have been analyzed as drug candidates for protozoan infectious diseases [3, 85, 86]. According to our data, the sulfated positions in the carbohydrates can be critical for the inhibitory quality [3]. Collectively, these studies highlight the possibility that carbohydrate drugs may be developed for the prophylaxis and treatment of parasitic infectious diseases. The results of our studies highlight the possibilities for countermeasures against malaria and toxoplasmosis [3, 85].

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A single mutation in the gatekeeper residue in TgMAPKL-1 restores the inhibitory effect of a bumped kinase inhibitor on the cell cycle

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ABSTRACT

Toxoplasma gondii is the causative pathogen for Toxoplasmosis. Bumped kinase inhibitor 1NM-PP1 inhibits the growth of *T. gondii* by targeting TgCDPK1. However, we recently reported that resistance to 1NM-PP1 can be acquired via a mutation in *T. gondii* mitogen-activated protein kinase like 1 (TgMAPKL-1). Further characterization of how this TgMAPKL-1 mutation restores the inhibitory effect of 1NM-PP1 would shed further light on the function of TgMAPKL-1 in the parasite life cycle. Therefore, we made parasite clones with TgMAPKL-1 mutated at the gatekeeper residue Ser 191, which is critical for 1NM-PP1 susceptibility. Host cell lysis of RH/ku80$^-$/HA-TgMAPKL-1^{S191A} was completely inhibited at 250 nM 1NM-PP1, whereas that of RH/ku80$^-$/HA-TgMAPKL-1^{S191Y} was not. By comparing 1NM-PP1-sensitive (RH/ku80$^-$/HA-TgMAPKL-1^{S191A}) and -resistant (RH/ku80$^-$/HA-TgMAPKL-1^{S191Y}) clones, we observed that inhibition of TgMAPKL-1 blocked cell cycle progression after DNA duplication. Morphological analysis revealed that TgMAPKL-1 inhibition caused enlarged parasite cells with many daughter cell scaffolds and imcomplete cytokinesis. We conclude that the mutation in TgMAPKL-1 restored the cell cycle-arresting effect of 1NM-PP1 on *T. gondii* endodyogeny. Given that endodyogeny is the primary mechanism of cell division for both the tachyzoite and bradyzoite stages of this parasite, TgMAPKL-1 may be a promising target for drug development. Exploration of the signals that regulate TgMAPKL-1 will provide further insights into the unique mode of *T. gondii* cell division.

1. Introduction

Toxoplasma gondii is the causative pathogen for Toxoplasmosis. It is a member of the Apicomplexans, which include several important pathogens, such as *Plasmodium, Cryptosporidium,* and *Neospora.* Without cell division, parasites cannot increase the parasite burden and cannot effectively disseminate throughout the host. Therefore, the cell division of parasites is essential to their life cycle. Protozoa in the Apicomplexa exhibit various types of cell division (Striepen et al., 2007). *Toxoplasma* and *Neospora* replicate via the two cell division process in the asexual stage, whereas *Plasmodium* species replicate by merogony (Arnot et al., 2011) in the blood stage. How parasites select these cell division types in each infection stage remains largely unknown.

The mitogen-activated protein kinase (MAPK) family functions in cell signaling to regulate cell division, cell differentiation, and stress responses in eukaryotic cells (Zhang and Liu, 2002). Genome analysis suggests that there are three MAPKs in the apicomplexan genome (Lacey et al., 2007). Api-MAPK2 and Api-MAPK3 are conserved among apicomplexans; however, Api-MAPK1 shares no homolog among *Plasmodium* species (Lacey et al., 2007). *T. gondii* encodes a single Api-MAPK1, *T. gondii* mitogen-activated protein kinase like 1 (TgMAPKL1) (TGME49_312570). Studies by Dr. Michael White group referred to TGME49_312570 as TgMAPKL1 and found that its similarity to mammalian MAPK is very low, being limited to the protein kinase domain. We also studied TGME49_312570 and, to avoid confusion, we changed our nomenclature of TgMAPK1 to TgMAPKL1 in agreement with the White group (personal communication).

We recently showed that TgMAPKL-1 appears to function in cell division (Sugi et al., 2013). Brown et al. also demonstrated that the protein kinase inhibitor SB505124, which directly targets TgMAPKL-1, arrests parasite cell division (Brown et al., 2014). Brumlik et al. further reported that parasites that expresses antisense RNA for TgMAPKL-1 have a slow growth rate and altered host cell

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signaling (Brumlik et al., 2013). Thus, inhibition of TgMAPKL-1 leads to parasite growth arrest, suggesting that TgMAPKL-1 has either a direct or indirect role in parasite replication. Although TgMAPKL-1 seems to function in parasite growth, the predicted genome sequence of *T. gondii* suggests that it lacks MAPKK and MAPKKK, which are upstream protein kinases for the MAPKs (Miranda-Saavedra et al., 2012).

Bumped kinase inhibitors (BKIs) represent a promising drug lead because they have little effect on mammalian protein kinases (Ojo et al., 2014a) but appear to be a potent inhibitors of parasite growth *in vitro* (Lourido et al., 2010; Murphy et al., 2010; Ojo et al., 2010; Sugi et al., 2010) and *in vivo* (Doggett et al., 2014; Lourido et al., 2013; Ojo et al., 2014b; Sugi et al., 2011). The primary targets of the BKIs are CDPK1s that carry a small gatekeeper residue, which makes the protein kinase sensitive to the BKIs. However, we recently showed that TgMAPKL-1 is the secondary target of the BKIs and that mutation of TgMAPKL-1 provides parasites with resistance to BKIs (Sugi et al., 2013). Ojo et al., (2014b) reported that BKI treatment of *Neospora caninum* inhibited the growth of the parasite in host cells – an effect that could not be explained as the result of CDPK1 inhibition because CDPK1 reportedly works in invasion and egress (Lourido et al., 2010; Sugi et al., 2010). Therefore, it is important to investigate how BKIs inhibit parasites by targeting the secondary target TgMAPKL-1. The investigation of the mode of action of bumped kinase inhibitor will help to reveal the atypical MAPK signaling pathway involved in the parasite life cycle.

In the present report, we employed chemical genetics to inhibit TgMAPKL-1 in an inducible manner. We used the bumped kinase inhibitor 1NM-PP1 and parasites in which the gatekeeper residue had been genetically mutated such that their susceptibility to this BKI was altered (Bishop et al., 2000). Similar chemical-genetics approaches were previously used to analyze other protein kinases in *Toxoplasma* (Donald et al., 2006; Lourido et al., 2010; Sugi et al., 2010) and *Plasmodium* (Ojo et al., 2014a). By using a parasite bearing TgMAPKL-1 with a small gatekeeper amino acid (BKI-susceptible) and a parasite bearing TgMAPKL-1 with a large gatekeeper amino acid (BKI-resistant), we could observe the effect of TgMAPKL-1 inhibition on parasite cell cycle progression. Here, we provide the first evidence that BKI affects parasite cell cycle progression by targeting TgMAPKL-1.

2. Materials and methods

2.1. Chemical reagents and antibodies

The following antibodies were used for Western blotting and immunofluorescence staining: an α-HA epitope tag rat monoclonal antibody (Roche, Basel, Switzerland), α-TgIMC3 rat antisera (Anderson-White et al., 2011), and an α-TgGAP45 rabbit polyclonal antibody (Plattner et al., 2008). 1NM-PP1 (Merck KGaA, Darmstadt, Germany) was dissolved in DMSO; ammonium pyrrolidinedithiocarbamate (PDTC), RNase A, and propidium iodide (Sigma-Aldrich, St. Louis, USA) were dissolved in distilled water.

2.2. Plasmids

To knock-in the gatekeeper mutated TgMAPKL-1 sequence in the native TgMAPKL-1 locus on chromosome XI, we produced a construct containing the 5'UTR from TgMAPKL-1, the HXGPRT selectable marker cassette, and the TgMAPKL-1 cDNA sequence fused with an N-terminal HA-epitope tag under the control of the GRA1 promoter sequence (Fig. 1A). Knock-in constructs for replacing the wild-type TgMAPKL-1 sequence in the chromosome with the gatekeeper-substituted TgMAPKL-1 expression cassette were made as follows: the HA-tag was amplified with primers HA_F and HA_R (Table 1) from pCMV-HA (Takara, Shiga, Japan) and inserted into the *Eco*T22I and *Eco*RI sites of pTgMAPK1-WT (Sugi et al., 2013). The resultant plasmid was designated as pHA-TgMAPKL-1, which encodes the TgMAPKL-1 expression cassette fused to the N-terminal HA-epitope. For the knock-in by homologous recombination, the 5'UTR of TgMAPKL-1 was amplified with the primers 5'UTR_F and 5'UTR_R (Table 1) from the genomic DNA of RH/ku80-/hxgprt- and inserted into the *Hin*dIII site of pHA-TgMAPKL-1 by using the InFusion cloning system (Takara). The resultant construct was designated as pKnock-In-HA-TgMAPK1. To substitute the gatekeeper residue, pKnock-In-HA-TgMAPK1 was PCR amplified with the primers F_GK_Ala and R_GK_35, or F_GK_Tyr and R_GK_35 (Table 1). The PCR fragments were ligated by using the InFusion cloning system.

2.3. Host cell culture

Human foreskin fibroblast (HFF) cells were maintained in the Dulbecco's modified Eagle medium (DMEM) (Life Technologies) supplemented with 25 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS). Vero cells were maintained in the DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 5% FCS. When the parasites were inoculated, DMEM (Nissui Pharmaceutical) with 1% FCS was used.

2.4. Transgenic parasites

The parasite strain RH/ku80$^-$/hxgprt$^-$ (Huynh and Carruthers, 2009) (ATCC: PRA319) was used as the parental strain of the transgenic parasites throughout this study. Parasites were maintained and serially passaged to new host Vero cells as described elsewhere (Roos et al., 1994). For transfection, plasmids were linearized with *Hpa*I and *Bsi*WI. Then, 2.5 μg of linearized DNA was introduced into 10^6 parasites by using Nucleofection with Nucleofector II (Lonza, Basel, Switzerland) as described elsewhere (Sugi et al., 2013). After selection with 25 μg/mL mycophenolic acid and 25 μg/mL xanthine, parasites were cloned by limiting dilution, and the resultant clones were subjected to PCR and PCR-RFLP analysis to screen for clones with the replaced chromosome.

2.5. Host cell monolayer disruption assay

Host Vero cells were seeded in 96-well plates at a density of 10^4/well and incubated for 24 h before parasite inoculation. Parasites were filter-purified, counted, and inoculated at a density of 3×10^3/well following incubation with the test concentration of 1NM-PP1 at room temperature for 10 min. Cells were incubated for 5 days with medium changes every 2 days. After this incubation, the infected host cells were fixed with methanol, stained with crystal violet, and then measured at OD_{600}. Average values from non-treated and mock-infected (control) wells were estimated to be 100%.

2.6. Cell cycle analysis

Cell cycle synchronization was performed by use of PDTC treatment as described elsewhere (Conde de Felipe et al., 2008). Briefly, parasites were inoculated into Vero cells at a multiple of infection 1.0 and incubated for 12 h before PDTC treatment. Infected cells were then treated with 80 μM PDTC for 8 h and then washed three times at 37 °C with warm medium to wash out the PDTC. After the washout, medium with or without 250 nM 1NM-PP1 was added and incubated extensively for 7 hours. Purified samples were prepared for flow cytometric analysis as described elsewhere (Conde de Felipe et al., 2008). Briefly, infected cells were washed with ice-cold phosphate buffered saline (PBS) three times to remove the extracellular parasites and were then harvested with scrapers. Host cells were ruptured by serial passage through a 25G needle three